

Inheritance of lethal gene 'luteus-Pa' from cacao progenies obtained by self and cross-fertilization

Herencia del gen letal 'luteus-Pa' en progenies de cacao obtenidas por autofecundación y fecundación cruzada

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ABSTRACT

Keywords:

Cacao
Self-cross pollination
Lethal factor
Mendelian segregation
Chi-square (X^2) test

With the aim to study the inheritance of lethal gene 'luteus-Pa' in three derivated progenies from self and cross-fertilization of cacao (*Theobroma cacao* L) varieties, a study was carried out during January to September, 2013. As genetic material C-25, S-5 y Pa-150 varieties were utilized and this one were self and cross pollinated and their progenies were divided in two replicates with 100 seeds each and sowed in plastic recipients containing a substrate 2:1 (soil: sand). In order to contrast the observed segregation with its expected homologous 3:1 (monogenic inheritance), the Chi-square (X^2) test was utilized. The results shown that only one from self-fertilized progeny of C-25 variety exhibited the mendelian segregation 3:1 in both replicates, so that it lead to infer this one is carrier of lethal gene 'luteus-Pa' in heterozigote condition whereas that the progenie from Pa-150 x C-25 cross no found none lethal seedling and submitted to Chi-square (X^2) test it could be to infer this one no segregate in the proportion 3:1 therefore, the Pa-150 variety is no carrier of this lethal gene. A better comprehension on photobiological origin, supported by molecular markers and the functional genomics of this mutant can help to identify and understand the factors involve in the photosynthetic mechanisms.

RESUMEN

Palabras clave:

Cacao
Auto-alopolinización
Factor letal
Segregación mendeliana
Prueba chi-cuadrado (X^2)

Con la finalidad de estudiar la herencia del gen letal 'luteus-Pa' en progenies derivadas de autofecundación y fecundación cruzada de tres variedades de cacao (*Theobroma cacao* L), se realizó este ensayo durante enero a septiembre, 2013. Como material genético se utilizó las variedades C-25, S-5 y Pa-150 que fueron auto y alopolinizados y sus progenies fueron divididas en 2 repeticiones de 100 semillas cada una y sembradas en contenedores plásticos conteniendo un sustrato 2:1 (suelo:arena). Para contrastar la segregación observada con la esperada 3:1 (herencia monogénica), se utilizó la prueba Chi-cuadrado (X^2). Los resultados muestran que sólo la progenie autofecundada de la variedad C-25 exhibió la segregación mendeliana 3:1 en ambas repeticiones, de modo que se puede inferir que esta variedad es portadora del gen letal 'luteus-Pa' en condición hererocigota; mientras que en la progenie de la cruce Pa-150 x C-25 al no encontrarse ninguna plántula letal y sometida a la prueba Chi-cuadrado (X^2), se infiere que falla en segregar en una proporción 3:1; por consiguiente, la variedad Pa-150 no es portadora de este gen letal. Una mejor comprensión sobre el origen fotobiológico apoyado por marcadores moleculares y la genómica funcional de este mutante, puede ayudar a identificar y comprender mejor los factores involucrados en el mecanismo fotosintético.

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Cacao (*Theobroma cacao* L.) is a species native to the tropical rain forests of South America whose beans are the basic input for the chocolate industry and derivatives, cosmetic and pharmaceutical industries. Wild and cultivated cacao populations exhibit genetic variability due to sexual reproduction and natural cross-pollination. The pressure of evolutionary forces, particularly, the mutations have given rise to recessive genes that have survived for generations in heterozygous condition. Mutations occur at very low frequencies modifying the genotype causing unfeasibility of the embryo or premature death of some seedlings. In cacao seedlings from Parinari series was observed leaf chlorosis, followed of necrosis and finally death of seedling, at approximately 30 to 40 days (Almeida *et al.*, 1998). It has been reported that Pa-30, Pa-121 and Pa-169 varieties when they were self or cross-fertilized and their progenies subjected to Chi-square (X^2) test, segregated in a ratio 3:1 (normal: lethal), inferring that are heterozygous genotypes carrying the lethal gene 'luteus-Pa' (Bartley *et al.*, 1983) and this gene is expressed only in recessive homozygous, being a character of monogenic inheritance (Bartley, 1969). Self-pollination or alopolinización between carriers' heterozygous trees produce a phenotypic ratio 3:1 (normal and lethal) to the emergency and a ratio 2:1 (2 normal heterozygotes and 1 dominant homozygote) to adulthood (Yamada, 1982). Another study using six varieties from Parinari series pollinated with Pa-169 variety (carrying the lethal gene 'luteus-Pa'), only Pa-44 variety showed segregation 3:1 verifying again monogenic inheritance (Yamada *et al.*, 2005). A physiological study of cacao seedlings carriers of lethal gene 'luteus-Pa', identified differences between normal and mutant seedlings at 15 days after sowing. Variations in growth and subsequent death of seedlings to 60 days were related to the depletion of reserves from cotyledons, inactivity of the photosynthetic apparatus and oxidative stress (Rehem *et al.*, 2011). Cacao as allogamous species, the lethal gene flow among populations can increase the frequency of this gene in the progeny and may become a limiting factor for mass propagation of seeds as rootstocks increasing production costs in the nursery and the inevitable replant new seeds. In some populations of common cacao exist heterozygous trees carriers of lethal gene 'luteus-Pa' who may have inherited from some ancient variety from Parinari series. The lethal gene 'luteus-Pa' may be useful for identifying seedlings belonging to the

Pa series, in order to determine relationships between genotypes constituting a potential genetic marker and a tool for paternity tests (Bartley, 2005; Rehem *et al.*, 2010). The aim of this study was to verify the type of inheritance of the 'luteus-Pa' gene in progenies resulting from self and cross fertilization of three varieties from Peruvian cacao.

MATERIALS AND METHODS

The study involved three places: EEA-Tulumayo, genebank cacao and deckhouse mesh of the Agronomy Faculty (Tingo María). The experimental soil consisted of a mixture of organic substrate of forest and sand (ratio 2:1). The internal temperature ranged from 22.2 to 25.6 °C at 08: 00h and from 28.4 to 34.5 °C at 14:00 pm with a brightness of 65% and 85% of relative humidity. The water requirements of the seedlings were satisfied with intraday irrigation. The genetic material consisted of three clones: S-5, C-25 and Pa-150, and this one had two components: selfing (S-5 \emptyset and C-25 \emptyset) and outcrossing (Pa-150 x C-25), utilizing eight pods from five trees for each clone. The seeds of the resulting progeny were planted in plastic containers and placed in the deckhouse mesh. The number of seeds per treatment was 200 (two replications of 100 seeds each one) sowed in four trays 25 seeds per container. For statistical analysis, the Chi-square test (X^2) was chosen in order to test hypotheses about the kind of segregation from resulting progeny to one or a few pairs of genes of Mendelian inheritance. Its formula is as follows:

$$X_c^2 = \sum_{i=1}^c \frac{(O_i - E_i)^2}{E_i}$$

where:

O_i = i-th observed data

E_i = i-th data expected

C = number of phenotypic classes

The X_c^2 value will be contrasted with the $X_{(0.05, 1 \text{ df})}^2$ value to test H_0 .

The methodology used in the self-pollinations and cross-pollination involved (i) the choice of flower bud and isolation. To this purpose, 20 clear plastic tubes properly packed were placed above the flower buds and fixed to the tree until anthesis and hand pollination, (ii) for the self-pollinations, pollen from a drawn flower on the same tree was applied and then it was covered with the protective tube for eight days to avoid contamination with foreign

pollen, and among eight to ten days the fruit was covered with a transparent plastic bag cutted in the bottom ends to evacuate transpired water and protect of the infection from fungi spores and (iii) cross-pollination, it was similar to that described for the self-pollinations, with the difference in the pollen donor parent. Pollen receptors flowers corresponded to Pa-150 variety and as pollen donors to C-25 variety. Monitoring the growth of fruits fertilized from self and cross pollination was performed every two weeks during six months. At harvest the fruits were labeled, seeds were washed with sawdust to remove the mucilage and then disinfected with Cupravit (5 g kg⁻¹ seed) just before sowing. The date of sowing

and seedling emergence, the identifying of emergent seedlings: normal and chlorotic (lethal) and intraday monitoring of chlorosis evolution until the senescence and death of mutant plants (lethal), were recorded.

RESULTS AND DISCUSSION

Chi-square test (X^2) for progenies from selfing

The Table 1, shows that the S-5 progeny resulting from self-pollination, no exhibited none lethal seedling, being all normal. The $X^2_c = 33.333$ value was higher to $X^2_{t(0.05, 1df)} = 3.841$ value, and as falls out of the region of H_0 acceptance, so we inferring this progeny not be adjust to ratio 3:1 (normal: lethal) of the expected Mendelian segregation

Table 1. Chi-square (X^2) test for cacao segregating progenies from selfing S-5 variety.

Phenotype	Segregation	Observed value (O)	Expected value (E)	O - E	(O - E) ² / E
Normal	3	100	75	25	8.333
Lethal	1	0	25	-25	25.000
	TOTAL	100	100		$X^2_c = 33.333$

and consequently, this genotype is not carrier of lethal gene 'luteus-Pa' in heterozygote condition.

Instead in Table 2, the progeny of the C-25 variety resulting from self-pollination, the segregation of seedlings: normal and lethal in a ratio 3:1 was observed. On average 100 emergent seedlings (replicate 1), 71 seedlings exhibited normal green colour (synthetizing chlorophyll) and 29

seedlings not exhibited (non-synthetizing chlorophyll). The $X^2_c = 0.853$ value was inferior to $X^2_{t(0.05, 1df)} = 3.841$ value and as falls out of the region of acceptance of H_0 , it can be concluded that observed segregation ratio in the resulting progeny from selfing C-25 variety, it fits the pattern of expected Mendelian segregation 3:1. Likewise, similar $X^2_c = 0.853$ values were obtained in the replicate 2. So, it can be inferred that C-25 variety is carrier

Table 2. Chi-square (X^2) test for cacao segregating progenies from selfing of C-25 variety.

Phenotype	Segregation	Observed value (O)		Expected value (E)	O - E		(O - E) ² / E	
		R 1	R 2		R 1	R 2	R 1	R 2
Normal	3	71	79	75	-4	4	0.213	0.213
Lethal	1	29	21	25	4	-4	0.640	0.640
	TOTAL	100	100	100		$X^2_c =$	0.853	0.853

R1= replicate 1; R2= replicate 2

of the lethal gene 'luteus-Pa' in heterozygote condition (LPip) and the lethal seedlings are of genotype recessive homozygote (lp lp).

Similar results of segregation of normal and lethal seedlings ("chlorophyll deficient mutants") in a phenotypic 3:1 ratio

from selfing have been reported for cacao varieties from Parinari series (Yamada, 1982; Bartley *et al.*, 1983), corroborating the monogenic inheritance of lethal gene 'luteus-Pa' and identifying to the C-25 variety as a carrier genotype of this lethal gene. We believe that C-25 may be inherited the 'luteus-Pa' gene from cultivar

Pa-169 already identified as carrier and this one might have participated in different crosses in cacao breeding programs in Brazil and whose hybrid seed mixed with others hybrids it would arrived in Peru in the late 80s or earlier decades from Brazil, being found in some farms of Huánuco, San Martín and Rio Apurímac-Ene valley. Lethal seedlings (“mutants deficient in chlorophyll”) die shortly after emergence because chlorophyll is essential for photosynthesis processes and also in the absorbance and transference light energy to the reaction centers within of the chloroplasts (Ning *et al.*, 2013). His acute deficiency causes stunted growth, senescence and subsequent death. Although the genetic mechanism is not known, it has been reported that the yellow-pale (lethal) phenotype could be due to a problem that arises in the membranes of thylakoid which is essential for the photosynthetic process (Reed *et al.*, 2014).

Chi-square test (X^2) for the progenies of cross-fertilization

The Table 3 show the results of the segregation of cacao seedlings from Pa-150 x C-25 cross. We can observe

there the absence of segregation of normal and lethal seedlings in a phenotypic ratio 3:1 and being all (100%) of normal green colour and 0% abnormal (yellowish). The $\chi^2_c = 33.333$ value was higher to $\chi^2_{(0.05, 1df)} = 3.841$ and as falls out of the H_0 acceptance region, we can conclude which this obtained progeny by cross-fertilization does not adjust to expected Mendelian segregation 3:1 (normal: lethal), rejecting the H_0 (3:1 ratio). These results allow us to infer that Pa-150 variety (Parinari series) does not carry the lethal gene ‘luteus-Pa’ but the C-25 variety does; otherwise, they would have appeared as lethal seedlings in a 25% (expected frequency). Reports from Brazil admit that not all varieties from Parinari series are carriers of this lethal gene ‘luteus-Pa’, with exception of the Pa-169 variety (Yamada, 1982; Bartley *et al.*, 1983). Generally recessive lethal alleles have variable frequencies in the populations and they can persist over a long period of time. Natural selection eliminates copies of allele when they occur in homozygote genotypes; however, recessive lethal alleles are suppressed by dominant alleles in heterozygote genotypes, and they evade natural selection; hence heterozygote carriers of

Table 3. Chi-square (X^2) test for segregating progenies of cacao of the Pa-150 x C-25 cross.

Phenotype	Segregation	Observed value (O)	Expected value (E)	O - E	(O - E) ² / E
Normal	3	100	75	25	8.333
Letal	1	0	25	-25	25.000
	TOTAL	100	100		$\chi^2_c = 33.333$

recessive lethal gene have a selective advantage in the natural selection process (Ryder *et al.*, 1999).

Physiogenetics of evolution of the chlorosis from lethal seedlings

Chlorophyll deficiency or albinism is perhaps the most easily mutant phenotype detectable in higher plants and in *Arabidopsis thaliana*. Among the various mutants of normal green leaves, e.g. Chlorina mutant (green-yellowing), the physiology and genetic mechanism is different from others mutants (Jarvis *et al.*, 2000). The effects of mutations on chloroplast development, photosynthesis, accumulation of chlorophyll b, and levels of uptake of light by chlorophyll a/b associated proteins, have been examined by Kosuge *et al.*

(2011). Conditional lethal plants have been obtained by inactivation of the ribulose-1,5-bisphosphatocarboxilasa-oxygenase enzyme catalyzing both oxidation and carboxylation of ribulose-1,5-bisphosphate resulting in uptake of O_2 , CO_2 release (photorespiration) and NH_4 (Migge and Becker, 2000). In the Figure 1, the evolution of lethal chlorosis in seedlings from selfing C-25 variety carrying the lethal gene ‘luteus-Pa’ is showed. There we can see the chlorotic leaf yellowing followed by necrosis, senescence and death of the seedlings in a period which range among 12 to 28 days after emergence. According to Rehem *et al.* (2011), the leaf yellowing is a symptom of senescence associated with chlorophyll degradation and catabolized by polyphenol oxidase, among others. When occur the accumulation of anthocyanins in the

senescent leaves, this is preceded by chlorophyll degradation (Díaz *et al.*, 2006). The persistence of the chlorophyll when the photosynthetic capacity is affected, increases susceptibility to oxidative stress induced by light in the leaf cells. A protective role of anthocyanins as sunscreens and as neutralizers for reactive oxygen species (ROS) in young expanded leaves and senescent leaves susceptible to damage by light, it has been suggested by Baker and Hardwick (1972). On the other hand, the 'shared pathway' approach to lethals gene identification, the complexity

and redundancy of metabolic pathways in plants may limit the strategy overall. The assumption here is that disrupting any portion of the complex proteic will result in lethality (Meinke *et al.*, 2008). Studies with molecular markers found that the level of polymorphism between Pa-30 and Pa-169, based on RAPD and SSR markers, was compatible with the average values usually found in genetic diversity studies of *T. cacao* (Rehem *et al.*, 2010). Data from ESTs identified genes associated with Rubisco, peroxidases, and other proteins and enzymes related to carbon assimilation, respiration, and

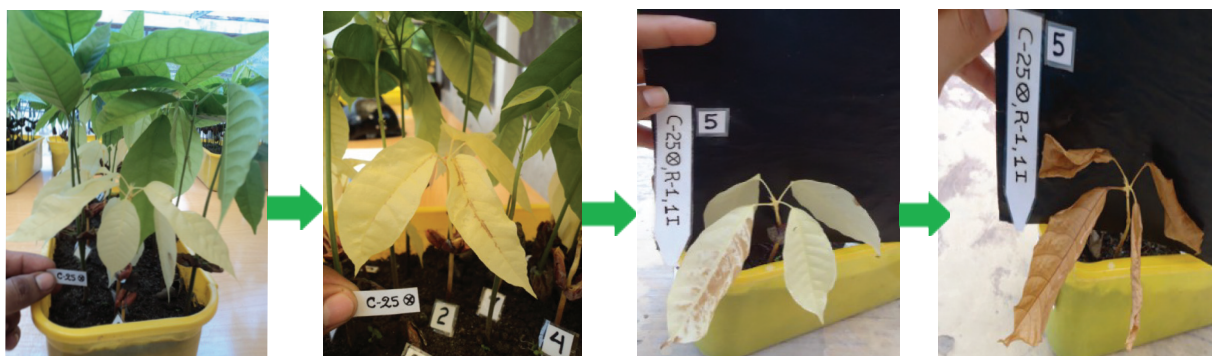


Figure 1. Evolution of the chlorosis of lethal seedlings from selfing C-25 variety.

photosystem 2. Mutant seedlings were characterized by synthesizing defective PsbO and PsbA proteins, which were overexpressed from 15 to 20 days after seedling emergence (Rehem *et al.*, 2016). A better understanding on the photobiological origin, the mapping with molecular markers and functional genomics of chlorophyll deficient mutants, can help to identify and understand the factors involved in the photosynthetic mechanism.

CONCLUSIONS

In the progenies from selfing, only C-25 variety showed a Mendelian segregation 3:1, identifying it as a heterozygote carrier of lethal gene 'luteus-Pa'.

The progeny from the crossing Pa-150 x C-25 does not give none lethal seedling failing in adjust to Mendelian segregation 3:1, therefore we can infer that the Pa-150 variety, is not carrier of lethal gene 'luteus-Pa'.

The physiogenetics basis of the chlorophyll deficient mutants are not yet very clear to understand the factors

involved in the genetic control, the photosynthetic mechanism and its photobiological origin.

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